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Use of conjugates of bovine serum albumin with poly(alkylene oxide)s for solubilization of riboflavin ester[1]

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Solubilization of 2',3',4',5'-tetrabenzoyl-5-acetyl-1,5-dihydroriboflavin (benzaflavin) by conjugates of BSA with poly(alkylene oxide)s [poly(ethylene glycol) and block co-polymers of ethylene oxide and propylene oxide (pluronics)] was investigated. Conjugates of BSA with pluronics were shown to have more solubilizing efficiency towards benzaflavin than BSA itself. Solubilized forms of benzaflavin are able to inhibit NADPH-dependent peroxidation of lipids in rat liver microsomes. A study of solubilized riboflavin ester transfer to mitochondria demonstrated that conjugates of BSA with pluronics may be advantageously employed for this purpose. Inhibitory properties of solubilized forms of benzaflavin were demonstrated by the study of their influence on the enzyme activity of D-amino-acid oxidase from pig kidney. The results show that solubilized forms of biologically active compounds based on conjugates of proteins with poly(alkylene oxide)s may be used for testing of chemical substances in biochemical systems.

In recent years, the mode of transfer of biologically active water-insoluble compounds to biological membranes has been extensively discussed as having important implications for the creation of new drug formulations. Colloidal dispersions of amphiphilic compounds {micelles of natural and synthetic surfactants [1], microemulsions [2,3], microspheres [4], nanogranules [5], liposomes [6] and microgels [7]} have been suggested as carrier systems.

The particle size is crucial with respect to the usefulness of various compositions because they have to pass through capillaries of less than 100 nm in diameter to ensure delivery of drugs to target cells. Therefore further studies are necessary to obtain novel small-sized transfer systems with sufficient capacity for water-insoluble compounds. The present paper reports the use of BSA conjugated with water-soluble poly(alkylene oxide)s [poly(ethylene glycol) (PEG)[3] and block co-polymers of ethylene oxide and propylene oxide (pluronics, proxanols)] as transport agents. These conjugates are star-shaped structures with a protein nucleus with

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[3] Abbreviations used: BF, benzaflavin (2',3',4',5'-tetrabenzoyl-5-acetyl-1,5-dihydroriboflavin) MDA, malonic dialdehyde (malonaldehyde); PEG, poly(ethylene glycol); POL, peroxidation of lipids.

polymer molecules attached, each at one site (Figure 1). Polymers of this type are soluble both in water and in organic solvents and may serve as catalysts for phase transfer [8]. Pluronics, which characteristically possess properties of polymeric amphiphilic surfactants, have also been shown to be inserted into biological membranes and to penetrate them [9,10]. This greatly accelerates pluronic-mediated phase transfer of hydrophilic compounds into cell membranes [11]. Covalent binding of pluronic chains to proteins results in conjugates capable of penetrating biological membranes [9].

Materials and methods

Materials

BSA was purchased from Serva, D-amino-acid oxidase from pig kidney and lactate dehydrogenase from pig skeletal muscle were purchased from Reanal; monomethoxypoly(ethylene glycol) [1] (M_r 1900) was purchased from Merck. Block co-polymers, obtained from the experimental plant MNPO 'NIOPIK' and with the following structures, were used:

(1) type RPE

$$C_4H_9O(\underset{\displaystyle CH_3}{CH}CH_2O)_{14}(CH_2CH_2O)H$$

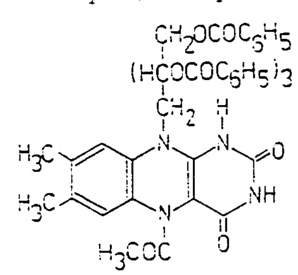
(2) type REP

$$C_4H_9O(CH_2CH_2O)_{20}(\underset{\displaystyle CH_3}{CH}CH_2O)_{14}H$$

The M_r of both co-polymers is ~2000; the content of propylene oxide is ~40%.

Monoaldehydes of these co-polymers were synthesized by the oxidation of end hydroxy groups [12].

2′,3′,4′,5′-Tetrabenzoyl-5-acetyl-1,5-dihydroriboflavin (benzaflavin; BF):



was purchased from the Moscow Experimental Vitamin Plant.

Synthesis of conjugates of BSA with poly(alkylene oxide)s

Conjugates of BSA with poly(alkylene oxide)s were synthesized by the interaction of protein ε-NH_2 groups with monoaldehyde derivatives of

poly(alkylene oxide)s by reductive-alkylation reaction at pH 9.0 in 0.02 M borate buffer at 20°C. The polymeric reagent/protein ratio was 50:1. The labile amidine groups were reduced with $NaBCNH_3$. Polymers that had not reacted were removed by gel-permeation chromatography with Toyopearl-40 as a support and water/ethanol (5:1, v/v) as an eluent. The control of polymer impurities was performed by using t.l.c. on Silufol plates in chloroform/ethanol/water (36:12:1, by vol.) Iodine vapour was used to stain the plates. The solutions were dialysed against water and freeze-dried. The protein was assayed by u.v. spectroscopy and the biuret reaction. The average number of polymer molecules coupled to one molecule of protein was calculated from the difference between the M_r value for unmodified and modified proteins, assuming a polymer M_r of 2000 (pluronic) and 1900 (PEG). Structures of conjugates are presented in Figure 1. Characteristics of these conjugates are given in Table 1.

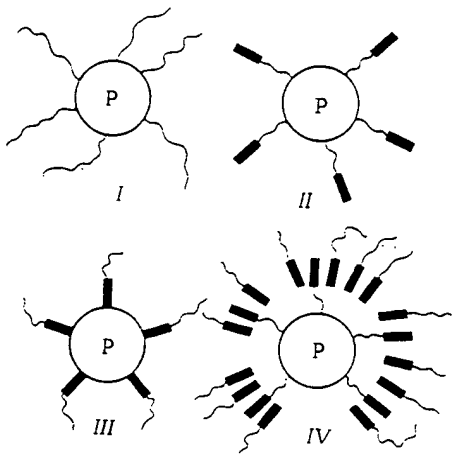


Figure 1

Structure of proteins (P) conjugated with poly(alkylene oxide)s. ▬, Hydrophobic block; ~~, hydrophilic block; *I*, BSA–PEG; *II* and *III*, BSA–pluronic; *IV*, BSA–pluronic + pluronic.

Table 1 Solubilization of BF and BSA and its conjugates with poly(alkylene oxide)s at 20°C

No.	Solubilizing agent	No. of polymer chains/protein molecule	Limit of solubilization (p)			s_{20} (S)
			pH 6.2 (method 1)	Water (method 2)	pH 7.2; 0.02 M phosphate buffer (method 2)	
1	BSA	—	2.4	6	7	4.6 ± 0.2
2	BSA–PEG (I)	14	5	11	21	4.2 ± 0.2
3	III	11	2	8	19	4.1 ± 0.1
4	II[a]	5	—	14	—	4.8 ± 0.2
5	II[a]	24	6	15	40	4.2 ± 0.1
6	IV[b]	44	10	15	—	3.6 ± 0.2
7	IV[b]	65	8	8	—	3.0 ± 0.1
8	IV[b]	100	3	6	13	2.8 ± 0.1

[a] The conjugate contains only covalently bound pluronic chains.
[b] The solubilizing agent was prepared from compound 4 by addition of free pluronic.

Study of BF solubilization by BSA and its conjugates with poly(alkylene oxide)s

BF solubilization was estimated using u.v. spectrophotometry and high-speed sedimentation analysis with an analytical ultracentrifuge (Spinco model E; Beckman) equipped with an absorption optical system, monochromator and photoelectric scanning system. Assays were carried out at 306 nm; the rotor speed was 48000 rev./min at 20°C. The determination of particle size was performed using the quasielastic light-scattering technique with an Autosizer L.C. (Malvern instrument).

Study of BF transfer to rat liver mitochondria

Rat liver mitochondria were isolated by the method described in [13]. The protein content was determined by the method of Bradford [14]. The amount of BF bound to mitochondria was determined photometrically by the difference between total BF content in solution and its content in the supernatant after incubation with mitochondria. This value reaches maximum after 1 min and remains constant during the following incubation with mitochondria. The incubation media was composed of 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 10 mM KH_2PO_4, 1 mM EDTA and 0.4 mg of mitochondrial protein in 1 ml (25°C).

The influence of BF on the processes of peroxidation of lipids (POL) in rat liver microsomes

Rat liver microsomes were obtained by the method described in [15]. POL was studied in 40 mM Tris/HCl buffer, pH 7.4, containing 0.2 ml of 5 mM NADPH, 0.2 ml of 50 μM Mohr's salt and 0.2 ml of 1 mM pyrophosphate; 0.2 ml of suspension containing rat liver microsomes and various volumes of conjugate solution were added to the assay solution. The volume of conjugate solution was calculated on the basis of the final concentration of BF. Pyrophosphate was dissolved in the solution containing solubilized BF in order to leave the total volume of incubation media unchanged compared with the control media (without conjugate). Incubation with constant shaking was for 30 min at 37°C. After incubation, the concentration of malonic dialdehyde (MDA, the final product of POL) was estimated by a standard method [16]. MDA was measured by reading the absorbance of its adduct with thiobarbituric acid (ε 1.56×10^5 $M^{-1} \cdot cm^{-1}$ at 532 nm).

Study of enzymic activity of D-amino-acid oxidase from pig kidney in the presence of solubilized forms of BF

Enzymic activity of D-amino-acid oxidase was measured by the lowering of absorbance of NADH at 340 nm in a coupled system, containing both D-amino-acid oxidase and lactate dehydrogenase from pig skeletal muscle and by the polarographic method using a Clark oxygen electrode. The concentration of D-amino-acid oxidase in the reaction media was 0.5 mg/ml and that of D-α-alanine 0.17 mM.

Results and discussion

Solubilization of BF by BSA–poly(alkylene oxide) conjugates

On the basis of the above-described properties of pluronics, it might be expected that coupling of amphiphilic molecules to carrier proteins, e.g. BSA, would result in an enhanced ability to solubilize hydrophobic compounds. This was illustrated in a study of solubilization of water-insoluble BF.

Dihydroriboflavin esters exhibiting a wide spectrum of biological activities are important both as long-acting vitamin B preparations and as metabolic drugs [17].

BF solubilization in aqueous solutions of BSA and its conjugates with poly(alkylene oxide)s was estimated using a u.v. method at 306 nm, a wavelength corresponding to maximum absorption of BF. Absorption coefficients were measured on the basis of a plot of absorbance versus concentration of BF solubilized by BSA ($\varepsilon = 4.9 \times 10^3$ $M^{-1} \cdot cm^{-1}$), conjugate of BSA with PEG ($\varepsilon = 4.7 \times 10^3$ $M^{-1} \cdot cm^{-1}$) and conjugate of BSA with pluronic ($\varepsilon = 3.5 \times 10^3$ $M^{-1} \cdot cm^{-1}$) (Figure 2).

The limit of solubilization (p), being eqivalent to the number of molecules bound to one molecule of the solubilizing agent, was estimated using high-speed sedimentation analysis at $\lambda = 306$ nm. The use of the sedimentation technique is based on co-sedimentation of a BF bound to protein or conjugate.

The two methods of BF solubilization employed in the present study were suspending dry material in aqueous solutions containing a solubilizing agent (method 1) and introduction of BF dissolved in ethanol into these systems (method 2). Examination of both systems using the quasi-elastic light-scattering technique showed that introduction of BF from ethanolic solutions resulted in the occurrence of small particles of the size of the conjugates (less than 30 nm) along with larger ones with a radius of 200–3000 nm, presumably corresponding to BF aggregates. Solubilization using the former technique (method 1) yielded only small particles. (<30 nm). To compare the two approaches, the systems containing BF and solubilizing agents of different types were subjected to centrifugation. Solubilization limit values of BF by BSA and its conjugates are presented in Table 1. BSA showed a marked capacity for solubilization of BF. This appears to be the first report indicating the presence of flavin-binding sites on the BSA molecule. However, experiments specifically designed revealed an inability of pluronics to solubilize BF.

Table 1 demonstrates that all conjugate-containing systems were characterized by higher p values as compared with those containing BSA; conjugate II, with hydrophobic regions located on the periphery of macromolecules, showed the highest limit of solubilization. However, method 2 allowed more BF to be introduced into a solubilization system, supposedly owing to a specific kinetic behaviour of the process. It should be emphasized that an increased number of polymer chains in the conjugate, due to a higher rate of substitution (samples 4 and 5), did not

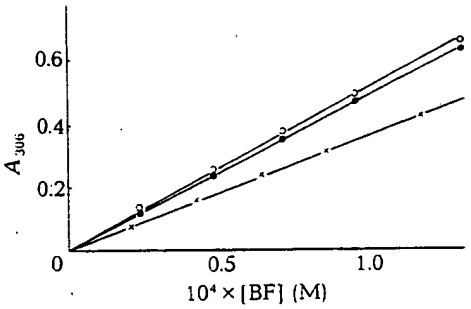


Figure 2

Solubilization of BF by BSA (○), conjugate of BSA with PEG (●) and conjugate of BSA with pluronic (×); conditions: 0.1 M Tris/HCl buffer, pH 8.3; 25°C; the concentration of BSA in the conjugate and free BSA in solution was 1 mg/ml.

appear to have any appreciable effect on the efficiency of solubilization. At the same time, a markedly enhanced density of polymer chains in the vicinity of the protein, which resulted from interactions of the introduced free pluronics with pluronic chains covalently bound with protein, led to a decrease in the limit of solubilization, probably owing to enhanced shielding of the sites responsible for BF solubilization. Optimum incorporation of BF into the conjugate occurred at pH 7.6–9.0 (Figure 3). The limit of solubilization decreased with increasing ionic strength of the phosphate buffer solution.

Experiments conducted to study temperature-dependent solubilization of BF and BSA and its conjugates revealed marked heterogeneity of the solubilization systems at 40°C which was not normally observed in solutions containing pure protein components. Certain portions of sedimentograms indicated mean sedimentation coefficients which were approximately twice as high as those intrinsic in protein components (Table 2). In such systems, BF could probably facilitate the 'bridging' of protein molecules to give rise to aggregates. Comparison of relative amounts of different particles in the experimental systems demonstrated that the proportion of aggregates in solutions containing BSA–pluronic conjugates was significantly lower than in all other BSA-containing systems. Polymer chains of these conjugates appear to be responsible for steric hindrance inherent in BF-mediated intermolecular interactions between proteins.

It is appropriate to surmise, on the basis of these findings, that BF molecules are localized in hydrophobic parts of the conjugated BSA. This inference is confirmed by the fact that attachment of pluronics to other proteins, e.g. IgG or α-chymotrypsin, which lack the ability to bind this ligand, had no effect on their solubilizing potency.

BF transfer from solubilized forms in rat heart mitochondria

The above data suggested the possibility of using conjugates of BSA with poly(alkylene oxide)s for the delivery of BF to target cells. Therefore, we investigated the transport of solubilized BF in biological systems, specifically BF transfer to rat liver mitochondria. This system demonstrates the relationship between the amount of bound BF and the concentration of free BF (BF_{free}) in the presence of either BSA or its conjugates. Quantita-

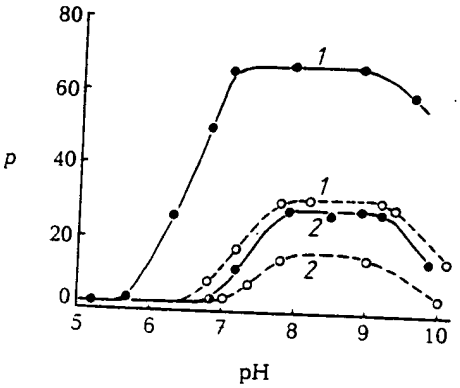


Figure 3

Relationship between BF solubilization limits (p) and pH (20°C; 0.2 M phosphate buffer). *1*, Visual method; *2*, sedimentation method; ---, BSA; ——, conjugate II.

Table 2 Temperature-dependent solubilization of BF by BSA and its conjugates (0.02 M phosphate buffer, pH 7.2)

No.	*Solubilizing agent (number of polymer chains in conjugate)*	*T (°C)*	*p*	*s_{20} (S) (solubilizing agent)*	*s_{20} (S) (BF complex)*	*Proportion of different forms (%)*
1	BSA	20	17	4.2	6.3	100
		40	17	6.7	11.0	47
2	BSA–PEG (14)	20	21	4.2	6.2	53
		40	21	6.5	6.6	100
					10.5	49
3	III (11)	20	19	4.2	6.8	51
		40	19	6.6	6.8	100
					13.2	47
4	II (24)	20	40	4.4	5.9	53
		40	40	6.8	10.1	100
					14.2	21
5	IV[a] (100)	20	18	2.9	7.3	79
		40	18	5.8	6.2	100
					12.0	80
					6.6	20

[a]Solubilizing agent was prepared from compound 4 by addition of free pluronic.

tive data on BF transfer are presented as binding isotherms in Figure 4. At the BF concentration interval studied, the plot of BF_{bound} versus BF_{free} is linear:

$$\frac{[BF]_{bound}}{[MT]} = K[BF]_{free}$$

where K denotes the coefficient of proportionality related to the chemical nature of the solubilizing agent and [MT] is the concentration of mitochondrial protein. The linear correlation between the two variables indicated that, for the experimental results characterizing initial portions of binding isotherms, K values were independent of the levels of the solubilizing agent. Thus the amount of BF bound by mitochondria may be

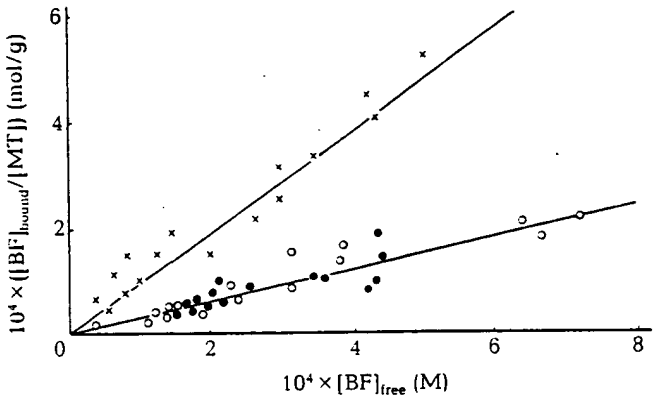


Figure 4

Relationship between concentration of bound BF (mol/g of mitochondrial protein) and concentration of free BF. Solubilizing agent: ○, BSA; ●, BSA–PEG; ×, BSA–pluronic (structure *II*). Abbreviation: [MT], concentration of mitochondrial protein.

calculated by the following simple formula:

$$\frac{[BF]_{bound}}{[MT]} = \frac{K[BF]_0}{(1 + K[MT])}$$

wherein $[BF]_0$ is total concentration of BF.

Also, Figure 4 shows the patterns which identify the ability of BSA and BSA–PEG to transfer BF to membranes. Both solubilizing agents may be characterized by the same K value: $K = 0.31 \pm 0.01$ (g of mitochondrial protein/litre)$^{-1}$. The higher transfer efficiency of BSA conjugate with pluronic for BF transport as compared with the BSA–PEG conjugate [$K = 0.96 \pm 0.01$ (g of mitochondrial protein/litre)$^{-1}$] is due to the weaker association between BSA–pluronic and BF solubilized by this conjugate. To summarize, BSA–pluronic conjugates have certain advantages over the remaining solubilization systems, both at the stage of solubilization and during transport of BF. This makes them useful tools for the development of new drug formulations.

Influence of BF on POL in microsomes of rat liver

Water-soluble forms of BF may be employed to investigate the molecular mechanisms of its action in model biochemical systems. It was shown [17] that the tetrabutyrate of riboflavin, a compound similar in structure to BF, inhibits POL in rat liver mitochondria, initiated by the addition of adriamycin, an antitumour agent. Based on these observations it might be expected that BF also would be able to inhibit POL. Quantitatively the process of NADPH-dependent POL and the influence of solubilized forms of BF on its rate was studied by measuring the concentration of MDA (final product of POL) in liver microsomes. In preliminary experiments the influence of BSA, PEG and pluronics on the rate of NADPH-dependent

POL was studied. BSA at a concentration up to 0.7×10^{-4} M and PEG at a concentration up to 4.1×10^{-4} M had no effect on the NADPH-dependent POL. On the basis of decreasing MDA production in the presence of solubilized BF forms, we may conclude that BF, solubilized by BSA and its conjugates with poly(alkylene oxide)s, inhibits the process of NADPH-dependent POL in rat liver microsomes. It should be noted that the inhibitory action of BF does not depend on the nature of the solubilizing agent. This is depicted in Figure 5, which shows the relationship between the extent of POL inhibition and the concentration of BF solubilized by BSA and its conjugates with poly(alkylene oxide)s. The concentration of BF corresponding to a 50% inhibition under the conditions studied is 0.5×10^{-4} M. These investigations demonstrate the ability of BF to inhibit the POL process in biological membranes.

Influence of BF on the enzyme activity of D-amino-acid oxidase from pig liver

In previous work [18–22] the inhibitory action of water-insoluble esters of dihydroriboflavin on D-amino-acid oxidase from pig liver was investigated using the system of hydrated reversed micelles of surface-active compounds {aerosol OT [bis-(2-ethylhexyl)sulphosuccinate sodium salt]} in organic solvent (n-octane). The alternative possibility is the use of solubilized forms of riboflavin esters, for instance, BF bound with BSA or its conjugates with poly(alkylene oxide)s.

It was shown in other experiments that the addition of BF dissolved in ethanol to the reaction mixture does not influence the enzymic activity of D-amino-acid oxidase. However, solubilized forms of BF do produce an inhibitory effect. Figure 6 shows the experimental values of the relative activity of D-amino-acid oxidase, V/V_0 (V_0 and V are the rates of enzymic reaction in the absence and in the presence of BF respectively), plotted against the concentration of BF. The inhibitory effect of BF does not depend on the nature of the solubilizing agent. It should be noted

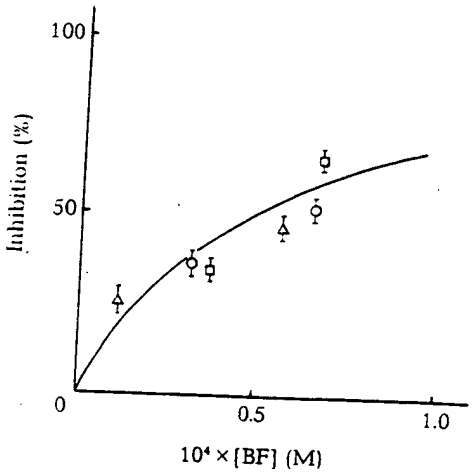


Figure 5

Relationship between degree of inhibition of MDA production in the process of NADPH-dependent POL in rat liver microsomes and concentration of BF, solubilized by BSA (○), conjugate BSA–PEG (□) and conjugate BSA–pluronic (△). Each experimental point is an average of six measurements.

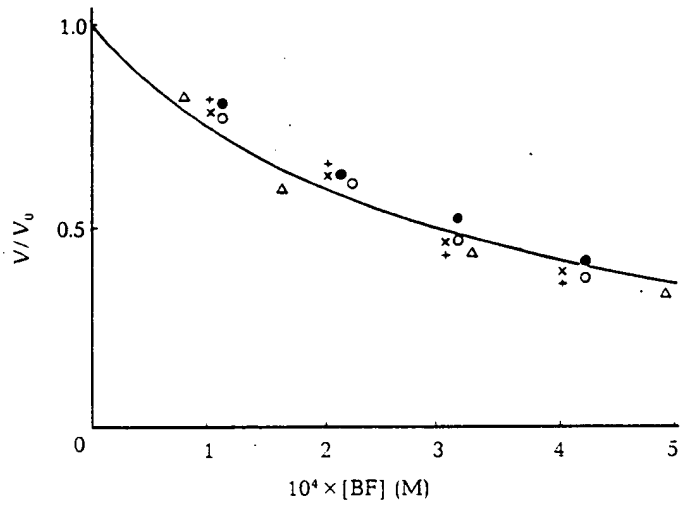


Figure 6

Inhibition of D-amino-acid oxidase by BF, solubilized by BSA (○ and ×), conjugate BSA–PEG (○ and +) and conjugate BSA–pluronic (△). The enzymic activity of D-amino-acid oxidase was registered by spectrophotometric (○, ●) and polarographic (×, △) methods. Conditions: 0.1 M Tris/HCl, pH 8.3, 25°C; the concentration of BSA in conjugates and free BSA in solution was 1 mg/ml.

that the course of the curve V/V_0 versus [BF] remains unchanged by alterations in the solubilizing agent concentration. In order to estimate the inhibitory ability of BF, the inhibition constant, K_i, was calculated as follows:

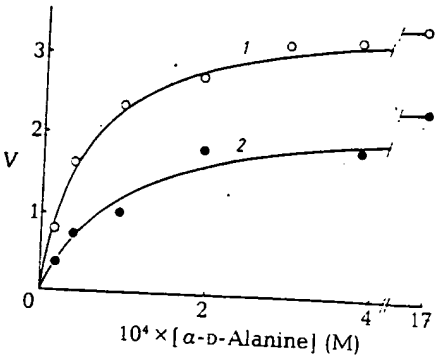
$$\frac{V}{V_0} = \frac{1}{1 + [I]/K_i}$$

where [I] is concentration of BF. The value of K_i appeared to be 0.29 ± 0.02 mM. It should be noted that the sensitivity of D-amino-acid oxidase entrapped in hydrated reversed micelles to the inhibition of BF is essentially higher: $K_i = 4.5 \times 10^{-5}$ M at ($[H_2O]/[$aerosol OT$] = 23$). This is because, in reversed micelles, the local concentration of BF in the vicinity of enzyme takes place as a result of entrapment of BF on the surface of the micelle.

Figure 7 shows the plot of the rate of the enzymic reaction catalysed by D-amino-acid oxidase versus the concentration of α-D-alanine in the absence of BF (○) and in the presence of 2×10^{-4} M BF, solubilized by the conjugate of BSA with PEG (●). Parameters of the Michaelis–Menten equation of experimental curves were calculated by the non-linear least-squares using algorithms by Marquardt [22] and Nelder and Mead [23]. In the absence of the inhibitor the Michaelis constant (K_m) and maximal rate (V_{max}) were found to be 4.9 ± 0.1 mM and 3.65 ± 0.05 ng-atom of O/min per mg of protein respectively. The decrease in the catalytic properties of D-amino-acid oxidase in the presence of BF is due both to a

Figure 7

Relationship between the rate of enzymic reaction catalysed by D-amino-acid oxidase (V) and the concentration of α-D-alanine in the absence (○) or presence of 2×10^{-4} M BF solubilized by a BSA–PEG conjugate (●). V is expressed in ng-atom of O/min per mg of protein.



decrease in the catalytic constant ($V_{max} = 2.5 \pm 0.1$ ng-atom of O/min per mg of protein) and to a decrease in the enzyme–substrate affinity ($K_m = 9.3 \pm 0.2$ mM).

Conclusion

The solubilization efficiency of BSA conjugates with poly(alkylene oxide)s [poly(ethylene glycol) and block co-polymers of ethylene oxide and propylene oxide (proxanols, pluronics)] for BF was studied. Conjugates of BSA with pluronics were shown to have greater solubilizing activity for this substance than the free protein. Solubilized forms of BF are able to inhibit NADPH-dependent POL in rat liver microsomes. The inhibitory action of BF does not depend on the type of solubilizing agent. The inhibitory properties were demonstrated by a study of the influence of solubilized forms of BF on the enzyme activity of D-amino-acid oxidase from pig kidney. Thus conjugates of BSA with pluronics are a new type of carrier for the delivery of biologically active water-insoluble compounds and their screening of biological systems.

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